Actions of S-(N-Methylthiocarbamoyl)-L-cysteine and Its Oxygen Analog on Pyridoxal Enzymes (Commemoration Issue Dedicated to Professor Eiichi Fujita on the Occasion of his Retirement)

AUTHOR(S):

kimura, Tomio; Esaki, Nobuyoshi; Tanaka, Hidehiko; Soda, Kenji

CITATION:

kimura, Tomio ...[et al]. Actions of S-(N-Methylthiocarbamoyl)-L-cysteine and Its Oxygen Analog on Pyridoxal Enzymes (Commemoration Issue Dedicated to Professor Eiichi Fujita on the Occasion of his Retirement). Bulletin of the Institute for Chemical Research, Kyoto University 1985, 63(2): 95-99

ISSUE DATE:

1985-08-24

URL:

http://hdl.handle.net/2433/77103

Bull. Inst. Chem. Res., Kyoto Univ., Vol. 63, No. 2, 1985

Actions of *S*-(*N*-Methylthiocarbamoyl)-L-cysteine and Its Oxygen Analog on Pyridoxal Enzymes

Tomio KIMURA*, Nobuyoshi ESAKI, Hidehiko TANAKA and Kenji SODA**

Received May 7, 1985

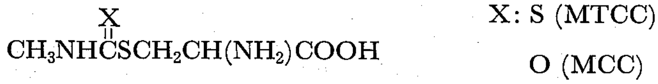
KEY WORDS: Suicide substrate for pyridoxal enzymes/
S-(*N*-Methylthiocarbamoyl)-L-cysteine/
S-(*N*-Methylcarbamoyl)-L-cysteine/

Considerable attention has been paid to the design of enzyme inactivators called enzyme suicide substrates. They are inherently unreactive, but are enzymatically converted into active species to inactivate the enzymes irreversible by a covalent modification of a catalytically essential amino acid residue or a coenzyme.[1] Therefore, the specificity of suicide substrates is based not only on their similarity to the normal substrate, but also on the mechanism of action of the target enzyme. Because of this high degree of specificity and efficiency, they are useful in the elucidation of the reaction mechanism and the physiological role of the enzyme as well as in the medical and agricultural uses.

Pyridoxal enzymes, which contain pyridoxal 5′-phosphate as a cofactor, catalyze racemization, transamination, decarboxylation and elimination of amino acids, and play an important role in the metabolism of amino acids. Their reaction mechanisms have been studied in detail in aspects of organic chemistry, and it has been clarified that pyridoxal enzymes can produce stable carbanion intermediates at α, β, or γ-carbon of substrate amino acids during their catalytic actions. Based on this reaction mechanism, various suicide substrates for pyridoxal enzymes such as 3-halogenated amino acids, vinylglycine and propargylglycine have been designed.[2] These compounds can be converted to intermediates containing a double bond conjugated with an imino group, and the intermediates react with a nucleophile at the active site as a good Michael acceptor to inactivate the enzyme.

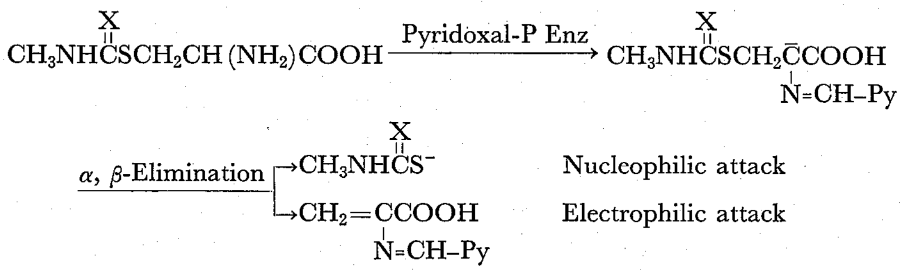
We have developed a novel suicide substrate for pyridoxal enzymes, *S*-(*N*-methylthiocarbamoyl)-L-cysteine (MTCC). L-MTCC is a unique amino acid in which *N*-methyldithiocarbamate binds to the β-carbon of L-alanine through a thioester linkage (Scheme 1). Since *N*-methyldithiocarbamate is a good leaving group (pKa of its conjugated acid=2.89),[3] MTCC may undergo α, β-elimination by pyridoxal

* Present address: Central Research Laboratory, Ube Industries, Ltd, Ube, Yamaguchi 755
** Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611



Scheme 1 MTCC and MCC

enzymes, which act on MTCC to abstract its α-proton, to form α-aminoacrylate intermediate and *N*-methyldithiocarbamate. α-Aminoacrylate intermediate is a well-known active species for the suicide inactivations of pyridoxal enzymes,[4] and *N*-methyldithiocarbamate is highly reactive as a potent nucleophile.[5,6] Therefore, MTCC acts most likely as a novel type of suicde substrate producing two active species as shown in Scheme II.



Scheme II Possible reactions.
Py: pyridine ring of pyridoxal 5′-phosphate (Pyriodxal-P)

With such expectation, we investigated the action of MTCC on two pyridoxal enzymes, amino acid racemase and methionine γ-lyase, and elucidated to be a suicide substrate for these enzymes.[7,8)9] We here describe the actions of MTCC on some other pyridoxal enzymes in comparison with its oxygen analog, *S*-(*N*-methylcarbamoyl)-L-cysteine (MCC) (Scheme 1).

MATERIALS AND METHODS

Materials. MTCC and MCC were synthesized from L-cysteine hydrochloride monohydrate by the reaction with methylisothiocyanate and methylisocyanate, respectively, according to the method of Smith et al.[9] Crystalline tryptophanase and crystalline tryptophan synthase ($\alpha_2\beta_2$ complex) of *Escherichia coli* were generous gifts from Dr. H. Yamada, Kyoto University and Dr. E. W. Miles, National Institutes of Health, U. S. A., respectively. The following enzymes were purified to homogeneity according to the procedures given in the literatures: amino acid racemase of *Pseudomonas putida*,[10] kynureninase of *Ps. fluorescens*,[11] methionine γ-lyase of *Ps. putida*,[12] and lysine ε-transaminase of *Flavobaterium lutescens*.[13]

Enzyme assay. The enzymatic α, β-elimination reactions of MTCC and MCC were followed by determination of pyruvate formed with lactate dehydrogenase. A standard assay mixture consisted of 80 mM potassium phosphate buffer (pH 7.5–8.5), 0.05 mM pyridoxal 5′-phosphate, 0.15 mM NADH, 100 units of lactate dehydrogenase (Sigma Type II, from rabbit muscle), MTCC or MCC, and enzyme in a final volume of 1.0 ml. In the case of tryptophan synthase ($\alpha_2\beta_2$), the assay mixture was sup-

plemented with 85 mM sodium chloride. The reaction was initiated by addition of enzyme, and carried out at 25°C. The reaction rate was monitored as a function of disappearance of NADH absorption at 340 nm. Ammonia was determined spectrophotometrically with glutamate dehydrogenase (Sigma Type II, from bovine liver) by the method of Tabor et al.[14] *N*-Methyldithiocarbamate and *N*-methylthiolcarbamate were determined by the colorimetric method of Gray and Streim.[15]

RESULTS

All pyridoxal enzymes tested (amino acid racemase, methionine γ-lyase, kynureninase, tryptophan synthase, tryptophanase and lysine ε-transaminase) acted on MTCC and MCC to produce an equimolar amount of pyruvate, ammonia and *N*-methyldithiocarbamate or *N*-methylthiolcarbamate. Pyruvate was converted to alanine with alanine dehydrogenase,[16] and identified by amino acid analysis. Ammonia was identified by the glutamate dehydrogenase method,[14] and *N*-methyldithiocarbamate and *N*-methylthiolcarbamate were identified by thin layer chromatography. This finding suggests that these compounds underwent α, β-elimination by the enzymes to form the carbamates corresponding to the β-substituents and the aminoacrylate intermediate, which is spontaneously hydrolyzed to pyruvate and ammonia. As shown in Table 1, the enzymatic α, β-elimination of MTCC by all enzymes tested is 2–8 fold faster than that of MCC. This appears to be mainly attributable to the fact that *N*-methyldithiocarbamate is better as a leaving group than *N*-methylthiolcarbamate.[5,17]

Table 1. Enzymatic α, β-elimination of L-MTCC and L-MCC and concomitant loss of enzyme activity

Enzyme	pH	Concn (mM)	Rel. rate[a] (MTCC/MCC)	$T_{1/2}$(min)[b]	
				MTCC	MCC
Amino acid racemase	7.5	10	2.2	4	10
Methionine γ-lyase	8.0	25	8.0	20	120
Kynureninase	8.5	2	3.4	2	8
Tryptophan synthase[c]	7.8	9	2.6	15	NI[d]
Tryptophanase	7.8	5	1.9	NI[d]	NI[d]

a) Relative rate represents the ratio of the elimination rate of L-MTCC to that of L-MCC under the reaction conditions indicated.
b) $T_{1/2}$ represents the time required to reduce enzyme activity to one half of the initial one. It was obtained from semilog plots for the pyruvate formation against time.
c) $\alpha_2\beta_2$ complex
d) No inactivation

All enzymes except for tryptophanase were observed to lose their eliminating activities time-dependently during α, β-elimination of MTCC. The decrease of the activities followed pseudo-first order kinetics. These pyridoxal enzymes can be classified four groups on the basis of elimination reactions of MTCC and MCC, and their inactivation reactions.

Amino acid racemase, methionine γ-lyase and kynureninase catalyzed α, β-elimination of MTCC and MCC with concomitant loss of their activities. The rates of α, β-elimination of MTCC by these enzymes are faster than those of MCC. Corresponding with the difference of elimination rates, the rates of activity loss during α, β-elimination of MTCC also are faster than those during MCC elimination (Table 1). Such dependency of the rate of activity loss on the elimination rate suggests that decrease of activity is caused by elimination products, either aminoacrylate intermediate or *N*-methyldithiocarbamate (or *N*-methylthiolcarbamate), or both of them.

Activity of lysine ε-transaminase was also reduced during α, β-elimination of MTCC and MCC. In this case, although α, β-elimination of MTCC proceeds 2 fold faster than that of MCC, the decreases of the enzyme activity by both compounds occur at the same time. This suggests that the mechanism of activity loss of this enzyme during α, β-elimination of MTCC or MCC may be different from those of enzymes mentioned above.

$\alpha_2\beta_2$ Complex of tryptophan synthase catalyzed α, β-elimination of MTCC and MCC. Although the same intermediate aminoacrylate must be formed in the reactions of both MTCC and MCC, the former reduced the enzyme activity, but the latter did not (Table 1). It is unlikely that the activity loss results from a nucleophilic substitution by an amino acid residue of the enzyme protein on β-carbon of MTCC before elimination reaction, because this enzyme is not inactivated by β-chloro-L-alanine[18] which must be more susceptible to such substitution than MTCC, owing to its β-substituent "Cl", a better leaving group than *N*-methyldithiocarbamate. Therefore, the activity loss may be due to *N*-methyldithiocarbamate released from MTCC, but neither aminoacrylate intermediate nor *N*-methylthiolcarbamate.

Tryptophanase catalyzed α, β-elimination of MTCC and MCC without any loss of activity (Table 1). This finding suggests that the enzyme has no residue for activity which can interact with either aminoacrylate intermediate, *N*-methyldithiocarbamate or *N*-methylthiolcarbamate.

DISCUSSION

The present results indicate that MTCC acts on pyridoxal enzymes such as amino acid racemase, methionine γ-lyase, kynureninase and tryptophan synthase ($\alpha_2\beta_2$ complex) to form pyruvate, ammonia and *N*-methyldithiocarbamate, and that during the α, β-elimination, the enzymes are inactivated. We reported the mechanisms of the inactivations of amino acid racemase and methionine γ-lyase by MTCC.[7,8] For amino acid racemase, both the isomers partitioned between racemization (127,500), elimination (506) and inactivation (1). This inactivation exhibited the characteristics of suicide inactivation, i.e., pseudo-first order kinetics, irreversibility, stoichiometric labeling of active enzyme by the C_3-chain derived from MTCC and protection by substrate.[7] L–MTCC also underwent the α, β-elimination by methionine γ-lyase and caused time-dependent enzyme inactivation. The partition ratio was about 800 per inactivation event. Also in this inactivation, aminoacrylate intermediate and *N*-methyldithiocarbamate was ascertained to act on the enzyme as active species.[8]

Kynureninase also seems to be inactivated by similar mechanism to methionine γ-lyase and amino acid racemase, based on the elimination and inactivation. The inactivation mechanisms of the other pyridoxal enzymes presented here which may be different from those of methionine γ-lyase and amino acid racemase are currently under investigation.

MTCC has been reported to inhibit the growth of *Escherichia coli*, *Streptococcus lactis* and *Lactobacillus arabinosus* more potently than MCC.[9] We have confirmed that MTCC also is more potent growth inhibitor than MCC against *Ps. putida* which produces amino acid racemase and methionine γ-lyase. Aminoacrylate, an intermediate expected seems to be active as an inactivator only in active site of the enzyme, because it is so unstable in water to be spontaneously hydrolyzed to pyruvate and ammonia. On the other hand, since *N*-methyldithiocarbamate is considerably stable at physiological pH,[3] it may act not only on the enzymes catalyzing its formation, but also on other enzymes. Therefore, MTCC probably exhibits the potent antibacterial activity *in vivo* as a bifunctional enzyme inhibitor which undergoes α, β-elimination by some pyridoxal enzymes to supply two different enzyme inhibitors, *N*-methyldithiocarbamate and aminoacrylate intermediate, and inactivates not only the pyridoxal enzymes but also other enzymes. Difference of antibacterial activities between MTCC and MCC may be explained, in part, by difference in stability and/or potency as a leaving group or nucleophile between *N*-methyldithiocarbamate and *N*-methylthiolcarbamate, substituents or elimination products.

REFERENCES

(1) R. H. Abeles and A. L. Maycock, *Acc. Chem. Res.*, **9**, 313 (1976).
(2) C. Walsh, *Tetrahedron*, **38**, 871 (1982).
(3) F. Takami, K. Tokuyama, S. Wakahara and T. Maeda, *Chem. Pham. Bull.*, **21**, 594 (1973).
(4) R. H. Abeles, "Enzyme-Activated Irreversible Inhibitors", eds. by N. Seiler, M. J. Jung and J. Koch-Weser, Elesevier/North-Holland, New York, 1978, p. 1.
(5) M. Bögemann, S. Peterson, O. E. Schultz and H. Söll, "Methoden der Organishen Chemie Vol. IX", eds. by E. Müller, O. Bayer, H. Meerwein and K. Ziegler, Georg Thieme Verlag, Stuttgart, 1955, p. 823.
(6) S. Oae, "Chemistry of Organic Sulfur Compounds Vol. I', Kagaku-Dohjin, Kyoto, 1968, p. 170.
(7) T. Kimura, N. Esaki, H. Tanaka and K. Soda, *Agric. Biol. Chem.*, **48**, 383 (1984).
(8) N. Esaki, T. Kimura, J. Goto, T. Nakayama, H. Tanaka and K. Soda, *Biochim. Biophsy. Acta*, **785**, 54 (1984).
(9) L. C. Smith, J. S. Humphrey, C. G. Skinner and W. Shive, *Texas Rep. Biol. Med.*, **21**, 296 (1963).
(10) K. Soda and T. Osumi, *Biochem. Biophys. Res. Commun.*, **34**, 363 (1969).
(11) K. Moriguchi, T. Yamamoto and K. Soda, *Biochem. Biophys. Res. Commun.*, **35**, 363 (1971).
(12) T. Nakayama, N. Esaki, K. Sugie, T. T. Beresov, H. Tanaka and K. Soda, *Anal. Biochem.*, **138**, 421 (1984).
(13) K. Soda and H. Misono, *Biochemistry*, **7**, 4110 (1968).
(14) C. W. Tabor, H. Tabor and U. Bachrach, *J. Biol. Chem.*, **239**, 2194 (1964).
(15) R. A. Gray and H. G. Streim, *Phytochemistry*, **52**, 734 (1962).
(16) T. Oshima and K. Soda, *Eur. J. Biochem.*, 100, 29 (1979).